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14. ABSTRACT Myc activation is common in breast cancer, correlated with triple negative disease, and associated with mortality. Thus, understanding Myc-driven breast cancer will facilitate knowledge of triple negative disease, a subtype of breast cancer with poor outcome and limited treatment options. Our laboratory has performed a genome-wide RNAi screen to identify genes that are required to tolerate Myc activation. Through this screen, we have identified Bud31, a poorly understood gene, and components of the fatty acid oxidation pathway (FAMs) as required for tolerance of Myc driven stress. Our goal herein is to test the hypothesis that Bud31 and FAMs may be putative new therapeutic entry points for Myc-driven breast cancer. Within this period of performance, we have confirmed the Myc-synthetic lethal phenotype of depletion of Bud31 and FAMs in our isogenic system and started to test the toxicity of genetic inhibition of these components in a panel of breast cancer cell lines. Furthermore, we have identified a FAM gene that is predictive of metastasis free survival in Myc high but not Myc low expressing breast cancer patients. In vivo studies planned for the following period will determine the significance and potential impact of these initial in vitro and in silico findings reported herein.					
15. SUBJECT TERMS breast cancer, Myc synthetic lethality, Bud31, fatty acid metabolism					
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## 1. INTRODUCTION:

*Background:* Myc activation is common in breast cancer, correlated with triple negative disease, and associated with mortality. Thus, understanding Myc-driven breast cancer will facilitate knowledge of triple negative disease, a subtype of breast cancer with poor outcome and limited treatment options. Myc confers both pro- and anti-tumorigenic effects on cells suggesting a sensitive balancing act for survival downstream of Myc activation. Supporting pathways, while not oncogenic by themselves, are necessary to help cells tolerate Myc driven stresses. Since direct pharmacological targeting of Myc has been shown to be largely unsuccessful, our laboratory has performed a genome-wide RNAi screen to identify supporting genes that are required to tolerate Myc activation in human mammary epithelial cells (Kessler et al.). Through this screen, we have identified BUD31, a poorly understood gene, and components of the fatty acid oxidation pathway (hereafter FAMs- fatty acid oxidation Myc synthetic lethal genes) as required for tolerance of Myc driven stress and as putative new therapeutic entry points for Myc-driven breast cancer. Our preliminary data indicate that BUD31 physically interacts with FAMs suggesting a functional relationship between the two. *Objective/Hypothesis:* Based on these preliminary data, we hypothesize that Myc activation confers a dependence on BUD31 and FAMs for breast cancer survival through a mechanism involving physical and functional interaction between BUD31 and fatty acid oxidation. *Specific Aims and Study Design:* To directly test the hypothesis above, we propose the following specific aims. AIM1: To determine if BUD31 interactions with lipid metabolism putative binding partners are necessary for the dependence of Myc-driven breast cancer on BUD31. We will perform structure-function analysis using the orthogonal methods of bimolecular fluorescence complementation and co-immunoprecipitation to confirm and map BUD31 interaction with lipid metabolism components and to test whether specific disruption of this binding alters the function of confirmed binding partners. AIM2: To confirm the Myc-induced reliance on FAMs in breast cancer and identify the mechanism of dependence of Myc-dependent breast cancer on fatty acid oxidation. We will test if depletion of FAMs in Myc dependent and independent cell lines induces toxicity in vitro. To identify Myc-driven changes to lipid metabolism, we will use mass spectrometry and Biolog analysis of metabolites. Analysis of publically available datasets for correlations between FAM gene expression and patient outcome will be performed. AIM 3: To systematically examine BUD31/FAMs requirements for Myc-driven breast cancer in vivo. We will utilize innovative in vivo screening technology and a panel of orthotopic syngeneic transplant mouse models both developed by our laboratory to test if BUD31/FAMs are necessary for tolerance of Myc. Genetic and pharmacological approaches to inhibit these targets will determine if this strategy presents a viable option for the treatment of Myc-driven breast cancer. *Impact:* Overall, this proposal identifies a novel therapeutic entry point for patients with Myc-driven and triple negative breast cancer. Our work will provide the pre-clinical data necessary for translating this strategy to the clinic with the ultimate goal of reducing both non-specific toxicities associated with treatment and overall mortality.

2. **KEYWORDS:** breast cancer, Myc synthetic lethality, Bud31, fatty acid metabolism

## 3. ACCOMPLISHMENTS:

**What were the major goals of this project? What was accomplished under these goals?**

Task 1: To determine if BUD31 interactions with lipid metabolism putative binding partners are necessary for the dependence of Myc-driven breast cancer on BUD31

We previously reported in our preliminary data that Myc activation in human mammary epithelial cells confers a dependence on Bud31 and similarly, Myc-dependent breast cancer cells lines, MDA MB 231 and SUM159, are highly sensitive to Bud31 depletion. To identify which regions of Bud31 are sufficient to rescue Bud31 function and protect against its depletion-induced growth inhibition, we generated a panel of Bud31 mutants. These stepwise 10 amino acid glycine mutants

and truncation mutants were used to test their ability to rescue growth after Bud31 depletion. SUM159 cells were dually manipulated to express both doxycycline-inducible shRNA targeting Bud31 and added back cDNA wildtype or mutant Bud31. We then performed competition assays in the absence or presence of doxycycline where infected cells (GFP+) were mixed with uninfected cells (GFP-) and the relative percent of infected cells was monitored. As shown in figure 1, Bud31 depletion has drastic effects on cell viability that can be rescued by wildtype Bud31, but not one of the zinc finger mutants. These data suggest that the zinc finger domain of Bud31 is critical for Bud31 function. Analysis of the complete panel of mutants is underway.

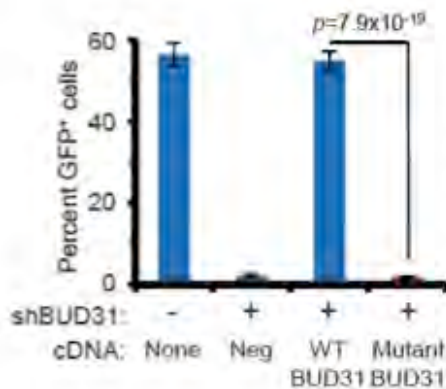


Figure 1. The zinc finger of BUD31 is required for rescue of BUD31 depletion-induced Myc synthetic lethality.

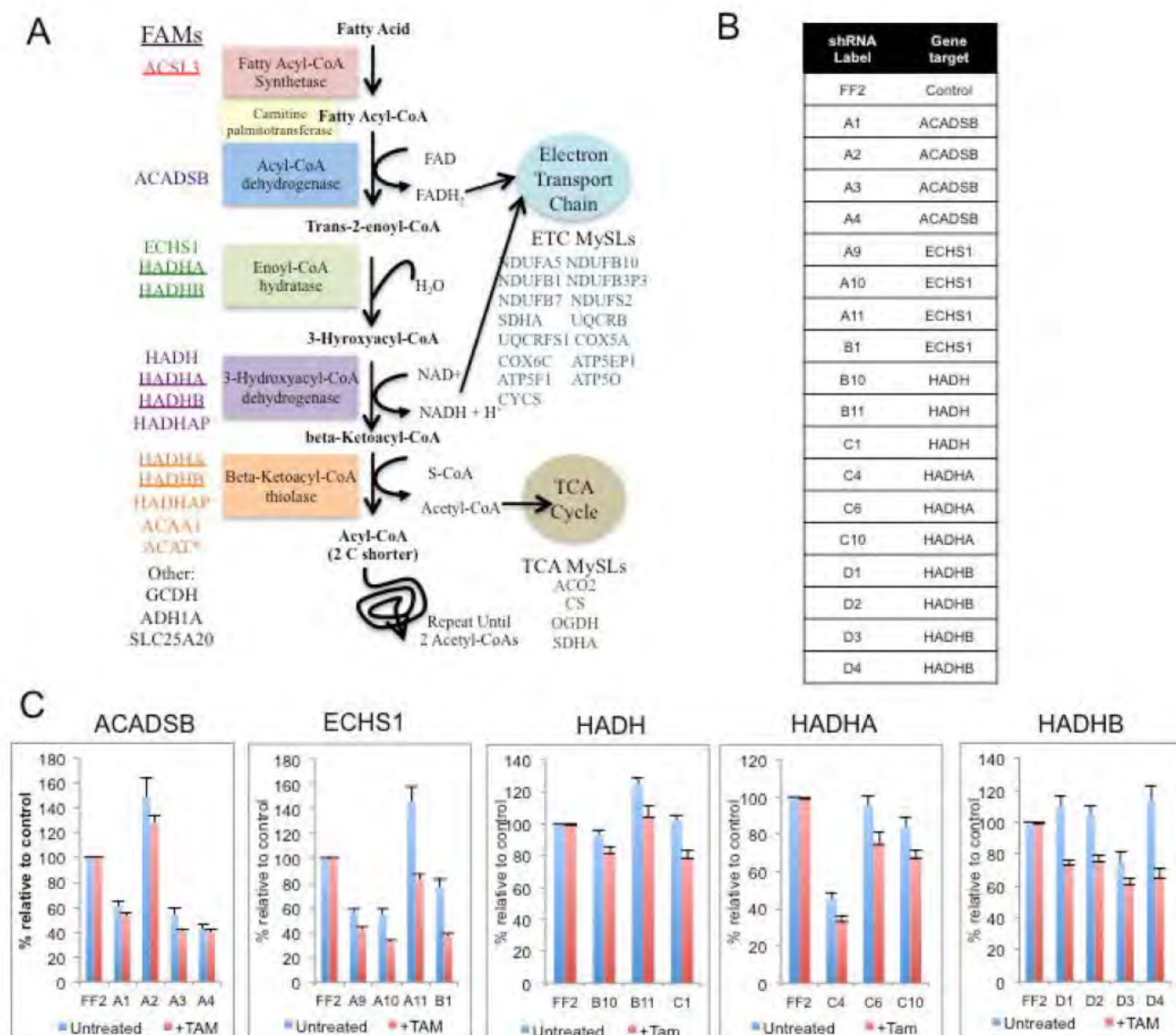
SUM159 cells were engineered with doxycycline-inducible shBud31 and constitutive expression of wildtype or zinc finger mutant BUD31 cDNA. These GFP-positive infected cells were then mixed with uninfected cells (80:20) and passaged twice in the presence or absence of doxycycline. Toxicity and rescue was assessed by flow cytometry for GFP.

Bud31 IP-MS data identified proteins involved in lipid metabolism as putative binding partners, as previously reported in our preliminary data. While we have yet to validate our IP-MS data using orthogonal methods (i.e. BiFC and Co-IP), the aforementioned mutant Bud31 constructs are available for mapping studies and we can use similar techniques to perform the converse functional analysis (i.e. mutant lipid metabolism partners). We have not yet started work on testing whether Bud31 depletion affects lipid metabolism candidate function since first, we need to validate the binding and second, we first want to test if depletion of the lipid metabolism candidates themselves altered breast cancer growth (task 2).

#### Task 2: To confirm the Myc-induced reliance on FAMs in breast cancer and identify the mechanism of dependence of Myc-dependent breast cancer on fatty acid oxidation

Our previously published whole genome screen (Kessler et al.) identified many genes involved in fatty acid metabolism and byproduct use as putative myc synthetic lethal genes, those whose depletion causes reduced cell viability or growth specifically in a myc active state (Figure 2). To confirm these candidates, we utilized our isogenic tamoxifen-activating Myc human mammary epithelial cells and expressed shRNAs targeting a variety of fatty acid metabolism genes. Figure 2 demonstrates that many shRNAs targeting did fatty acid metabolism genes did confer a myc synthetic lethal phenotype (e.g. ECHS1 shB1, HADH shC1, HADHB shD4), while others were straight lethal (e.g. ACADSB shA4). The difference between pure toxicity (reduced cell numbers in basal or myc activated states) and a myc activation specific phenotype, may simply involve potency and dosing of the shRNA of interest. To address this, we would need to clone the shRNAs of interest into our pINDUCER vector that allows for doxycycline regulation of depletion. Indeed, we have found that we must titrate down the expression of the most robust, efficient shRNA against Bud31 to induce a level of Bud31 depletion that is Myc synthetic lethal rather than just generally toxic (data not shown). These findings are compatible with potential clinical translation since therapeutics designed to reduce function rather than completely ablate its function are feasible.





**Figure 2. Validation of Myc synthetic lethal fatty acid metabolism candidates**

A. Mapping of FAMs to fatty acid oxidation (FAO) pathway and downstream utilization of by-products. This diagram depicts steps of FAO including enzymes (rectangles), metabolites, and usage pathways of by-products (ovals). FAMs mapping to FAO by Kegg pathway or Reactome are shown to the left: FAM = fatty acid metabolism MySLs; underlined = FAM and in BUD31 IP-MS data; \*only found in BUD31 IP-MS data. FAMs found in byproduct pathways are noted under the pathways. B. List denoting multiple unique shRNAs used to deplete fatty acid metabolism genes. C. Fatty acid metabolism candidate genes are Myc synthetic lethal. Human mammary epithelial cells engineered to express tamoxifen-inducible activation of Myc were further engineered to express shRNAs targeting putative Myc synthetic lethal. After control or tamoxifen treatment, cells were imaged daily with high throughput microscopy. Data are shown are 5 days of tamoxifen treatment and are normalized to untreated or tamoxifen treated control shRNA expressing cells.

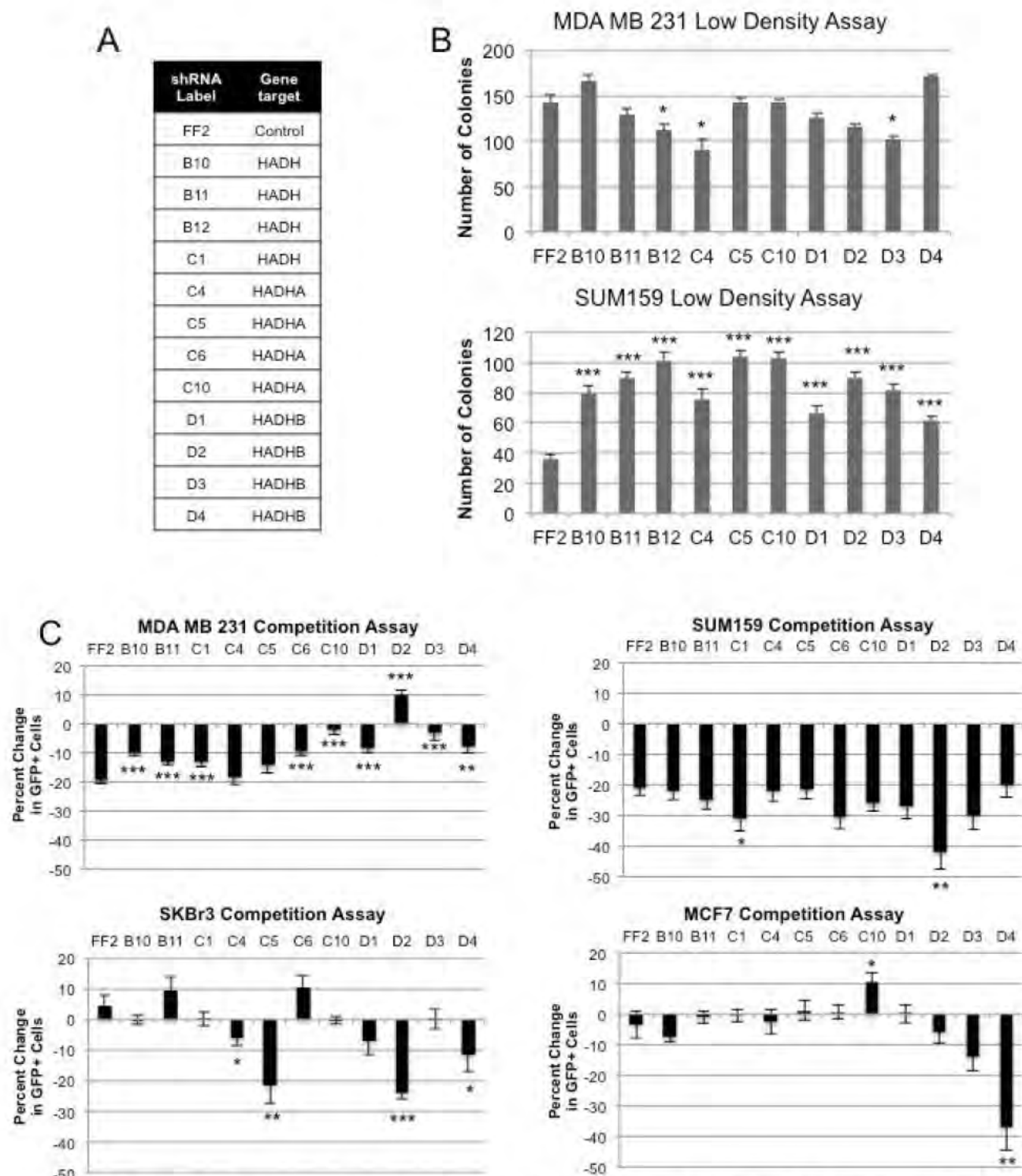
Since we were able to confirm the Myc synthetic lethal phenotype with fatty acid candidates in our isogenic system, we next wanted to know if depletion of fatty acid metabolism components affected myc-driven breast cancer. Our hypothesis was that genetic disruption of the fatty acid metabolism pathway would specifically reduce the growth potential of Myc-driven breast cancer, but not those that do not depend on Myc. We focused our attention on the genes comprising a trifunctional heterocomplex (HADHA, HADHB, HADH) that mediates three of the steps of fatty acid

oxidation, positing that disruption of this complex would serve to disrupt the entire pathway. To test our hypothesis, myc-dependent (MDA MB 232 and SUM159) and Myc-independent (SKBr3 and MCF7) breast cancer cell lines were engineered to constitutively express shRNAs targeting HADHA, HADHB, and HADH and then assayed for effects on growth and viability using low density assays and in vitro competition assays. As shown in figure 3, one shRNA against HADHA, HADHB, or HADH in MDA-MB231 was able to reduce colony formation in low density assays, however these same genetic manipulations exhibited better growth than control lines in competition assays. On the other hand, SUM159 cells grew better in low density conditions with candidate depletion compared to control, while exhibiting growth reduction in competition assays. The conflicting results observed with the same cell line in different assays and the inconsistencies between effects in two myc-dependent cell lines makes interpretation of the results challenging. The simple/surface interpretation of these data is to support the null hypothesis, that fatty acid oxidation is not a viable therapeutic intervention point for myc-driven breast cancer. However, given the molecular complexity of each of these lines, it seems premature to make that conclusion based upon only two myc-dependent cell lines. As a complementary approach, we queried two publically available databases of with genome wide RNAi libraries examined for toxicity across multiple cell lines (Cowley et al., Marcotte et al.). Cell lines with valid ATARIS scores for myc expression (Shao et al.), were sorted based on toxicity scores after myc depletion. In figure 4, the heat map depicts cell lines sorted left to right for highest to lowest sensitivity to myc depletion and then scored for response to depletion of the given shRNA targeting different lipid metabolism genes. ANOVA analyses revealed no difference between lipid metabolism genes' score in Myc depletion lethal and Myc depletion non-responsive groups thus suggesting that lipid metabolism depletion induced toxicity does not correlate with myc dependency. It is important to note, that these cell lines are cultured in different growth media often with supraphysiological levels of glucose, thus complicating interpretation of depletion of metabolism components. Ultimately, we care most about the response of breast cancer cells to fatty acid metabolism in a physiological relevant context. Experiments enumerated in task 3 will be the ultimate test for the viability of targeting fatty acid oxidation as a therapeutic intervention point for myc-driven breast cancer.

To test whether correlations exist between fatty acid/lipid metabolism gene expression and distant metastasis free survival of breast cancer patients, we analyzed the database described in Kessler et al.. As previously described, this data set is a compilation of nine datasets of patient survival stratified into Myc high and low cohorts. Shown in figure 5 are the tabular results for Cox-proportional hazards regression analysis for probes to fatty acid and lipid metabolism genes identified in our screen and with adequate signal and spread. Consistent with the data suggesting that HADHA is myc synthetic lethal, HADHA expression is correlated with patient outcome in the Myc high but not low dataset. Interestingly, HADHA expression correlated inversely with survival, a finding that is counterintuitive to the predicted outcome for a myc synthetic lethal phenotype. Furthermore, this is exactly the opposite from what is observed in neuroblastoma data sets where a high proportion of patients have high N-myc amplification: high expression of HADHA correlates with better survival in a patient data set (Zirath et al.).

Finally, for task 2, to explore whether Myc expression had a significant effect on cellular lipid profiles, we performed untargeted lipidomics in collaboration with Dr. Sreekumar's laboratory on human mammary epithelial cells following Myc activation at two and four days relative to control. Lipidomic analysis identified 14 lipid species that correlated significantly with Myc activation (Figure 6). Among the identified lipids there is a strong enrichment of triacylglycerol (TAG) species; however this may be a product of technical bias as there were significantly more TAGs detected than any other lipid species. Furthermore, assuming that 5% of all identified species may be significantly correlated by chance, our findings suggest there are fewer observations than expected and thus we cannot reject the null hypothesis. To address this we would need to be pursue targeted lipidomics methods to attempt to validate our initial findings and determine if there is indeed a strong relationship between MYC activity and the lipid species we have identified here.

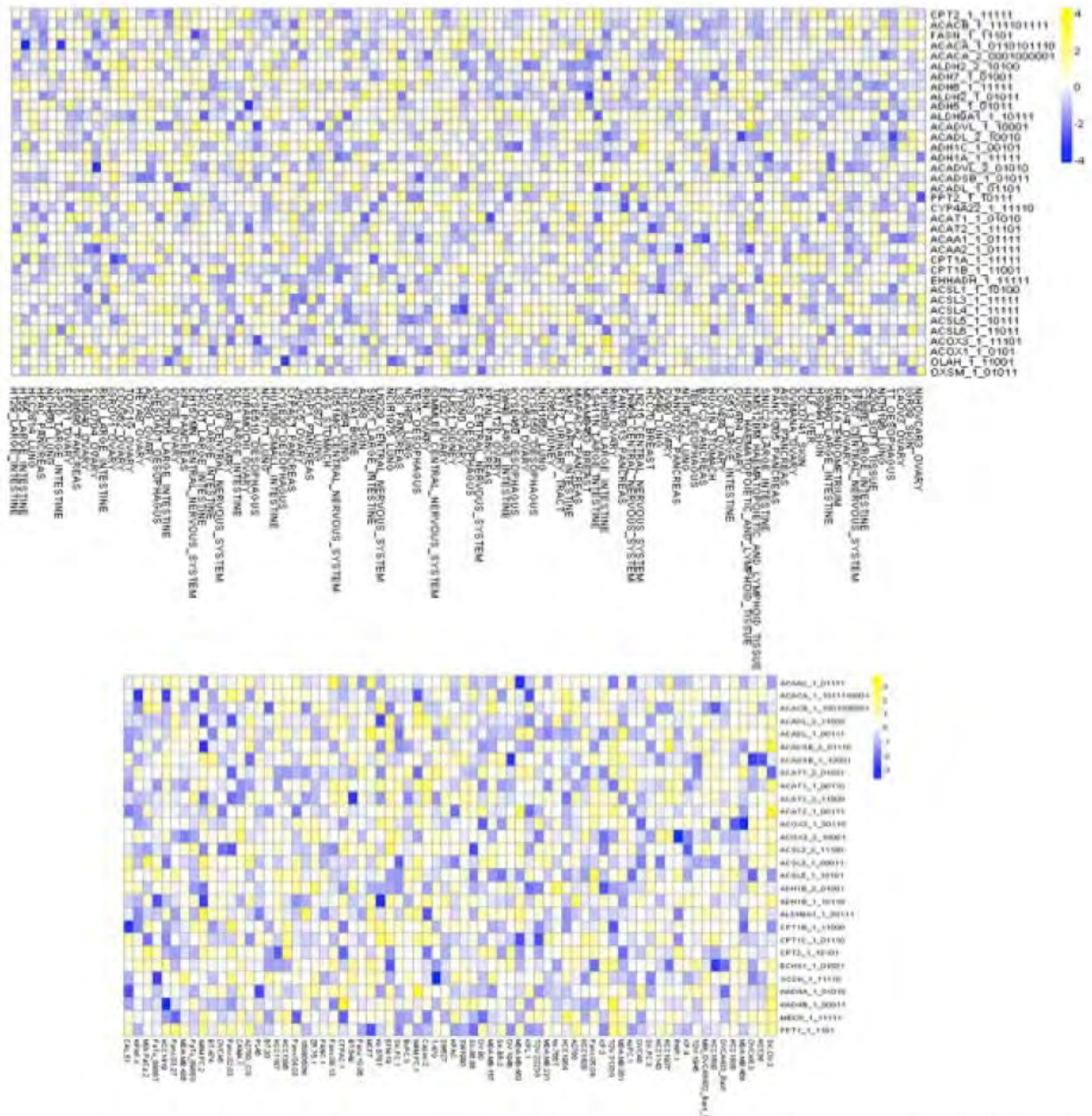




**Figure 3. Fatty acid metabolism candidate depletion has varying effects on Myc-dependent and Myc-independent breast cancer cell line growth in vitro.**

Myc-dependent (MDA MB 231 and SUM159) and Myc-independent (SKBr3 and MCF7) human breast cancer lines were tested for their dependency on validated Myc synthetic lethal fatty acid metabolism candidates. A. List denoting multiple unique shRNAs used to deplete fatty acid metabolism genes. B. Low density assays: Human breast cancer cells expressing control shRNAs or those targeting HADHA, HADHB, and HADH were plated at low density and allowed to grow until countable colonies were present in the control line. Average of total colony count from four replicates are shown. C. Competition assays: Breast cancer cell lines were infected with virus from pGIPZ expressing shRNA and GFP. Infected cells were mixed with uninfected cells (60:40), passaged at least three times, and monitored by flow cytometry for changes to population GFP percentages. Statistical analysis was performed using T-tests between control and shRNA of interest. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

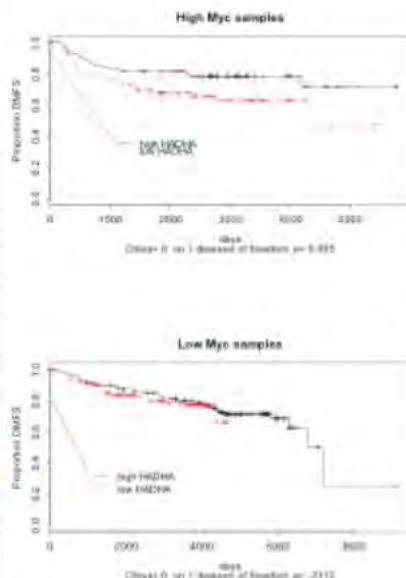




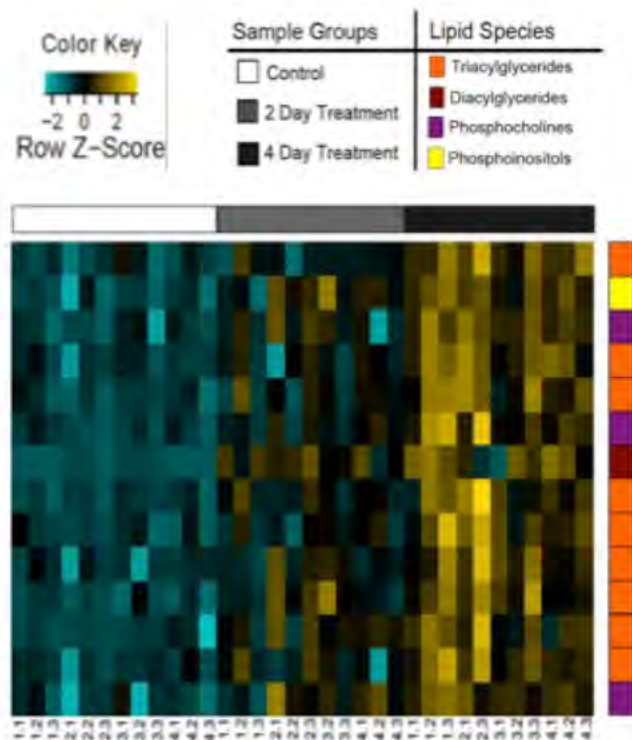
**Figure 4. Effects of depletion of fatty acid metabolism genes does not correlate with myc dependency across a large panel of human cancer cell lines.** Shown in the top heat map, Achilles V 2.0 dataset with ATARIS gene level scores from 102 cell lines passing quality control was obtained from the Broad's Project Achilles, and all the shRNAs relevant to fatty acid metabolism (FAM) were extracted from it. Myc ATARIS gene level score was used as indicator of Myc lethality, and cell lines were stratified according to it (most to least myc dependent sorted from left to right). ANOVA analyses revealed no difference between FAM genes' score in Myc depletion lethal and Myc depletion non-responsive groups. Shown in the bottom heat map, similar analysis was done with gene-level scores (GARP scores for ~15K genes across 72 cell-lines) from DPSC-Cancer database. ANOVA analyses revealed no difference between FAM genes' score in Myc depletion lethal and Myc depletion non-responsive groups.



Gene Symbol	Myc Low	Myc High
ACACA	0.6945	0.7927
ACSL3	0.4049	0.0759
ALAS1	<b>0.0013</b>	0.1259
CHD9	0.7686	0.2016
ECHS1	0.4475	0.6454
FASN	0.15	0.3372
FHL2	0.0848	0.5556
GPD2	0.9732	0.0688
HADH	0.7391	0.8022
<b>HADHA</b>	<b>0.2312</b>	<b>0.005</b>
HADHB	0.1368	0.5227
SLC25A20	0.8804	0.9763
TBL1X	<b>0.0169</b>	0.2293



**Figure 5. HADHA expression correlates with metastasis free survival in Myc overexpressing breast cancer patients.** The table lists the p values for Cox-proportional hazards regression analysis for probes with adequate signal and spread in a previously described compendium of breast cancer patient data (Kessler et al., 2012). Briefly, datasets from nine different publications were normalized and survival analyses was performed on high-myc expressed and low-myc expressed subcohorts. Metastasis free survival plots for patient groups stratified by Myc status and HADHA expression are shown.



**Figure 6. Lipid profiling downstream of Myc activation.** HMECs engineered to activate Myc in response to tamoxifen were treated for zero, two, or four days with tamoxifen then subjected to lipidomic profiling via a ABSciEx TripleTOF 5600 mass spectrometer with ESI running in MS/MS full scan mode. Samples were run in technical triplicate and biological quadruplicate in both positive and negative mode. Scan mode data was analyzed using LipidView software to identify and quantify relative abundances of lipid species. Relative abundances of lipid species were normalized relative to internal standards. To determine which species were significantly altered upon treatment, a regression model was applied to identify species which significantly changed in a linear relationship between control, day 2 and day 4 of treatment, which were then visualized using the ggplot2 package (Wickham, 2009) in the free statistical software R. Of the 930 lipid species assessed, only the 14 significantly altered species are shown in the heat map.

### Task 3: To systematically examine BUD31/FAMs requirements for Myc-driven breast cancer in vivo.

We have achieved internal IACUC approval for these experiments from Baylor College of Medicine and are actively pursuing ACURO approval. No animal experiments have been started since ACURO approval is still pending. However, our laboratory has made significant strides in library generation and other technology development that will expedite these experiments once animal use approval is in place.

#### References:

Cowley et al., Scientific Data 1, Article number: 140035 (2014). Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of context-specific genetic dependencies

Kessler et al. Science. 2012 Jan 20;335(6066):348-53. A SUMOylation-dependent transcriptional subprogram is required for Myc-driven tumorigenesis.

Marcotte et al., Cancer Discov. 2012 Feb;2(2):172-89. Essential gene profiles in breast, pancreatic, and ovarian cancer cells.

Shao, et al., Genome Res. 2013 Apr;23(4):665-78. ATARiS: computational quantification of gene suppression phenotypes from multisample RNAi screens.

Zirath et al., Proc Natl Acad Sci U S A. 2013 Jun 18;110(25):10258-63. MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells.

#### **What opportunities for training and professional development has the project provided?**

##### Training during Year 1:

As discussed in the training plan proposed for this postdoctoral fellowship, I have continued to participate in and attend both Rosen and Westbrook laboratory meeting, journal clubs relevant to breast cancer, breast disease research group seminars, BCM Career development seminars, and a variety of other breast cancer-relevant seminars at Baylor College of Medicine and MD Anderson Cancer Center. These interactions have provided continued acquisition of knowledge of the breast cancer field and opportunities for professional development including honing communication skills and networking. I have also learned new techniques such as high throughput microscopy while polished previously known skills such as cloning and flow cytometry. While I have yet to complete an official biostatistics class, I am gaining training in bioinformatics through interactions with fellow trainees and professors, especially for analysis of data shown in figures 4-6. Through collaborations with the Sreekumar laboratory, I am continuing to get a new understanding of both metabolic pathways and how to assess these pathways.

##### Symposia and Conferences attended during Year 1:

Annual Presidential Career Symposium- Feb. 26<sup>th</sup>, 2014

BCM Breast Center Research Meeting (poster presented- awarded 2<sup>nd</sup> place)- Sept. 11-12th, 2014

MD Anderson Metastasis Research Center Retreat- Dec. 5<sup>th</sup>, 2014

AACR Special Conference: MYC: from biology to therapy – Jan. 7-10, 2015

Dan L. Duncan Cancer Center (poster presented)- Jan. 26<sup>th</sup>, 2015

##### Publications during Year 1:

Cell Rep. 2014 Nov 20;9(4):1318-32. doi: 10.1016/j.celrep.2014.10.011. Epub 2014 Nov 6. The oncogenic STP axis promotes triple-negative breast cancer via degradation of the REST tumor suppressor. Karlin KL<sup>1</sup>, Mondal G<sup>2</sup>, Hartman JK<sup>3</sup>, Tyagi S<sup>3</sup>, **Kurley SJ**<sup>3</sup>, Bland CS<sup>3</sup>, Hsu TY<sup>1</sup>, Renwick A<sup>4</sup>, Fang JE<sup>5</sup>, Migliaccio I<sup>6</sup>, Callaway C<sup>7</sup>, Nair A<sup>3</sup>, Dominguez-Vidana R<sup>1</sup>, Nguyen DX<sup>8</sup>, Osborne CK<sup>6</sup>, Schiff R<sup>6</sup>, Yu-Lee LY<sup>9</sup>, Jung SY<sup>5</sup>, Edwards DP<sup>7</sup>, Hilsenbeck SG<sup>10</sup>, Rosen JM<sup>9</sup>, Zhang XH<sup>11</sup>, Shaw CA<sup>4</sup>, Couch FJ<sup>2</sup>, Westbrook TF<sup>12</sup>.

**How were the results disseminated to the communities of interest?** Nothing to report

**What do you plan to do during the next reporting period to accomplish the goals?** The statement of work describes the plan of action for accomplishing year 2 goals.

**4.IMPACT:** Nothing to report. Our findings are too preliminary to know the impact. By next Year 2 annual report we should have a better understanding of the impact of our ongoing work.

**5. CHANGES/PROBLEMS:**

**Changes in approach and reasons for change:** Given the inconsistent findings in the breast cancer cell lines assayed in vitro (figure 3) and our bioinformatic analysis of a large panel of cell lines (figure 4) when genetically targeting fatty acid metabolism, we are concerned with the viability of this strategy for treatment of breast cancer. However, metabolism is a complex set of networks and feedback systems that are highly influenced by context. As such, we propose that the best test will be the in vivo experiments proposed in task 3. If the proposed genetic perturbations do not significantly alter breast cancer growth in vivo, we will need to discuss a statement of work change as identifying the molecular mechanism of a less than robust phenotype seems inconsistent with the overall objectives put forth for this grant and funding agency.

**Actual or anticipated problems or delays and actions or plans to resolve them:** As described above, we are currently experiencing technical problems with our lipidomics approach which has delayed a portion of task 2. We are continuing our collaboration with members of the Sreekumar laboratory, who are working to address these technical issues.

**Changes that had a significant impact on expenditures:** Nothing to report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:** Nothing to report

**6. PRODUCTS:** Nothing to report

**7. PARTICIPANTS&OTHER COLLABORATING ORGANIZATIONS:**

**What individuals have worked on the project?**

Name:	Sarah Kurley, PI
Project Role:	PI
Nearest person month worked:	12
Contribution to Project:	Dr. Kurley has executed and guided data described herein. Training tasks listed in this postdoctoral fellowship were also completed by Dr. Kurley.

**Has there been a change in active other support?** Nothing to report

**What other organizations were involved as partners?** Nothing to report

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to report

**8. SPECIAL REPORTING REQUIREMENTS:** Not applicable

**9. APPENDICES:** No appendices.